

Fig. 1

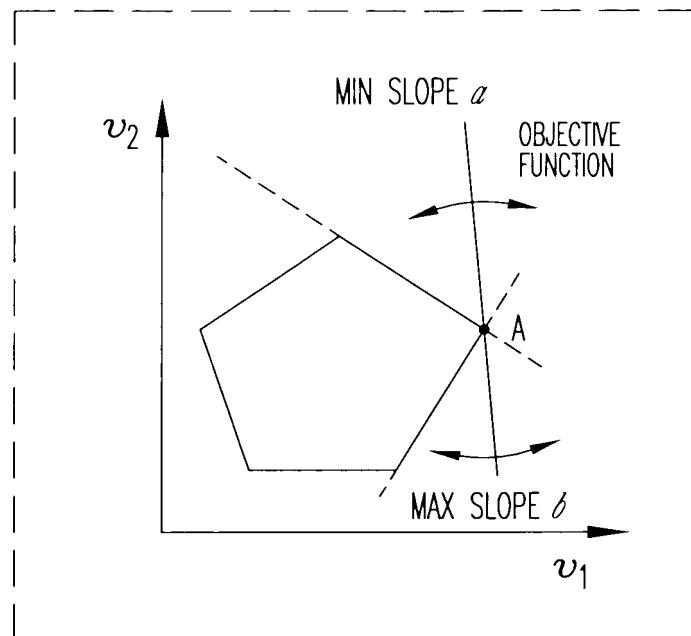


Fig. 2A

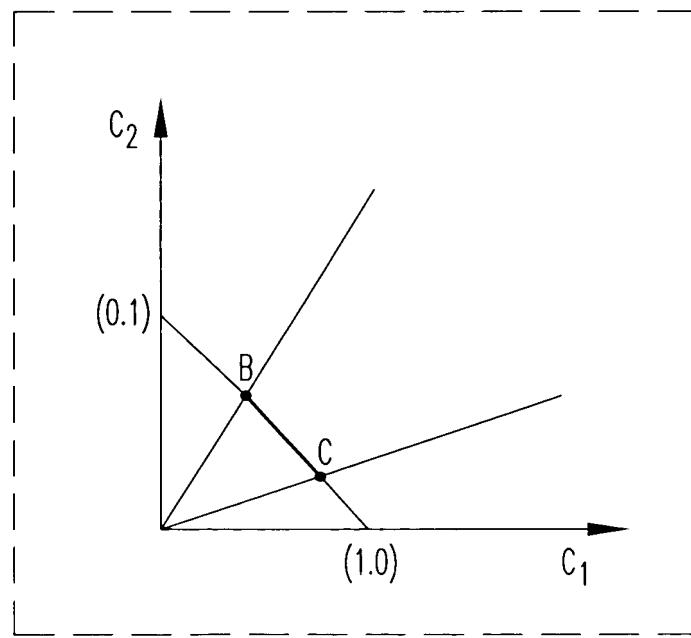


Fig. 2B

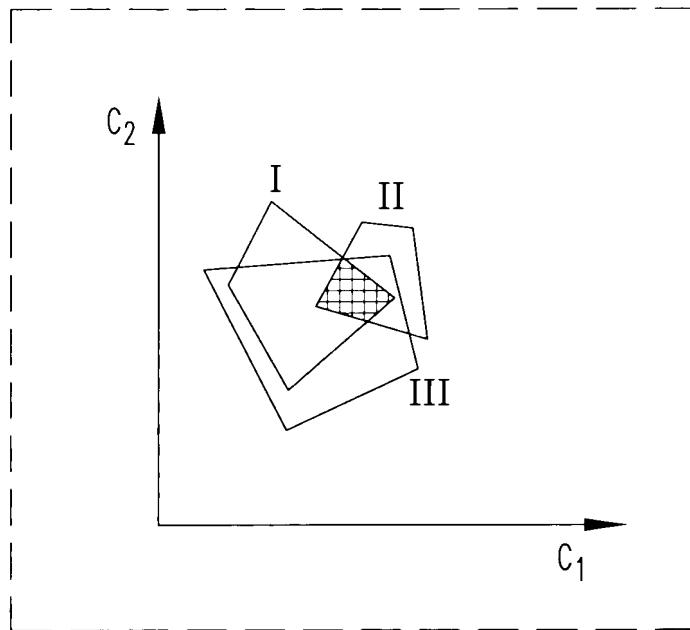


Fig. 3A

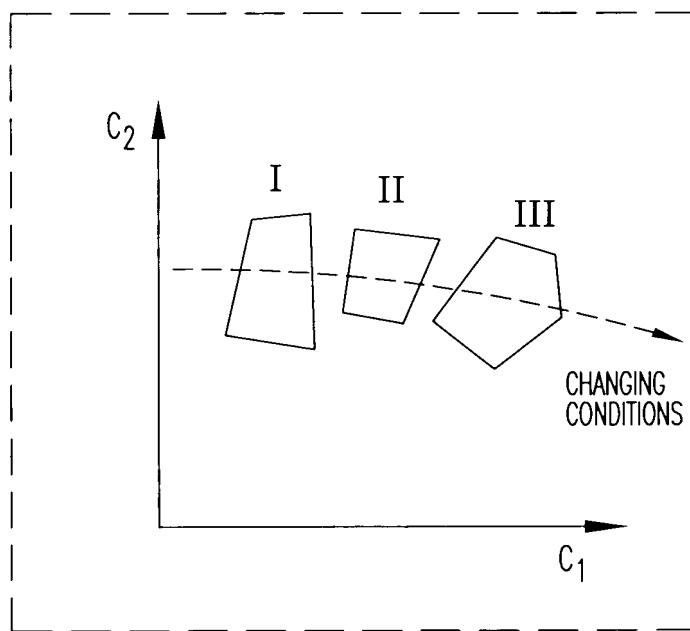


Fig. 3B

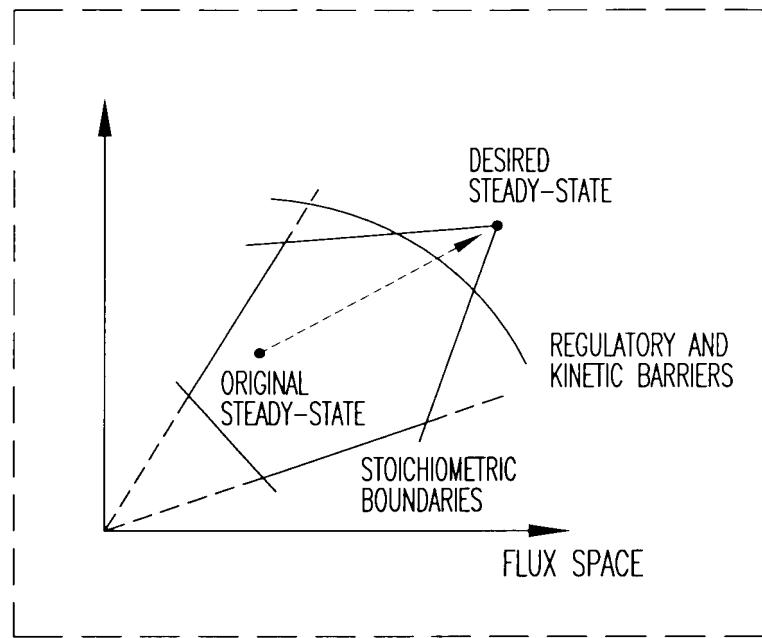


Fig. 4

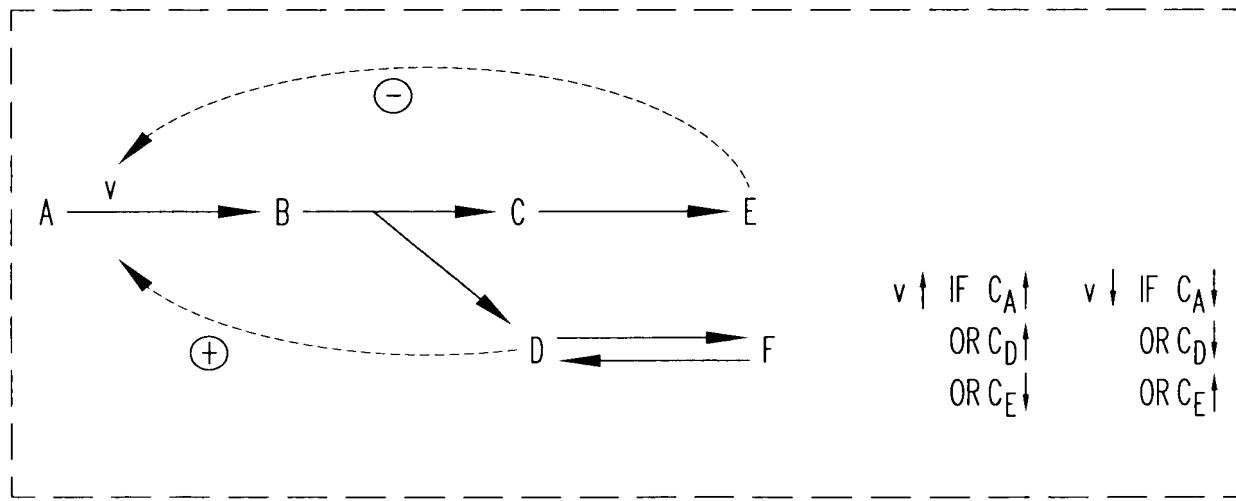


Fig. 5

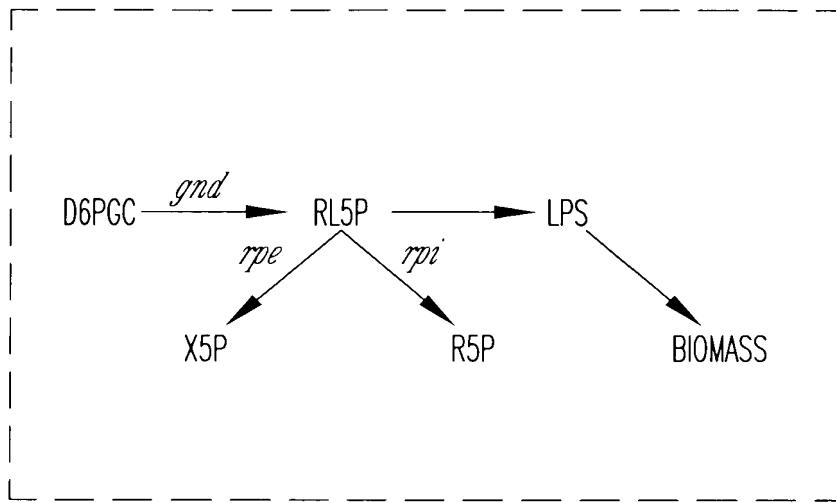


Fig. 6A

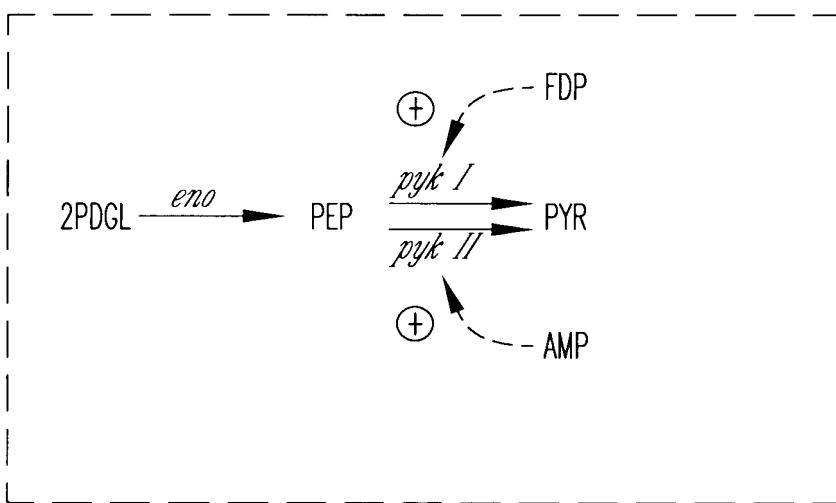


Fig. 6B

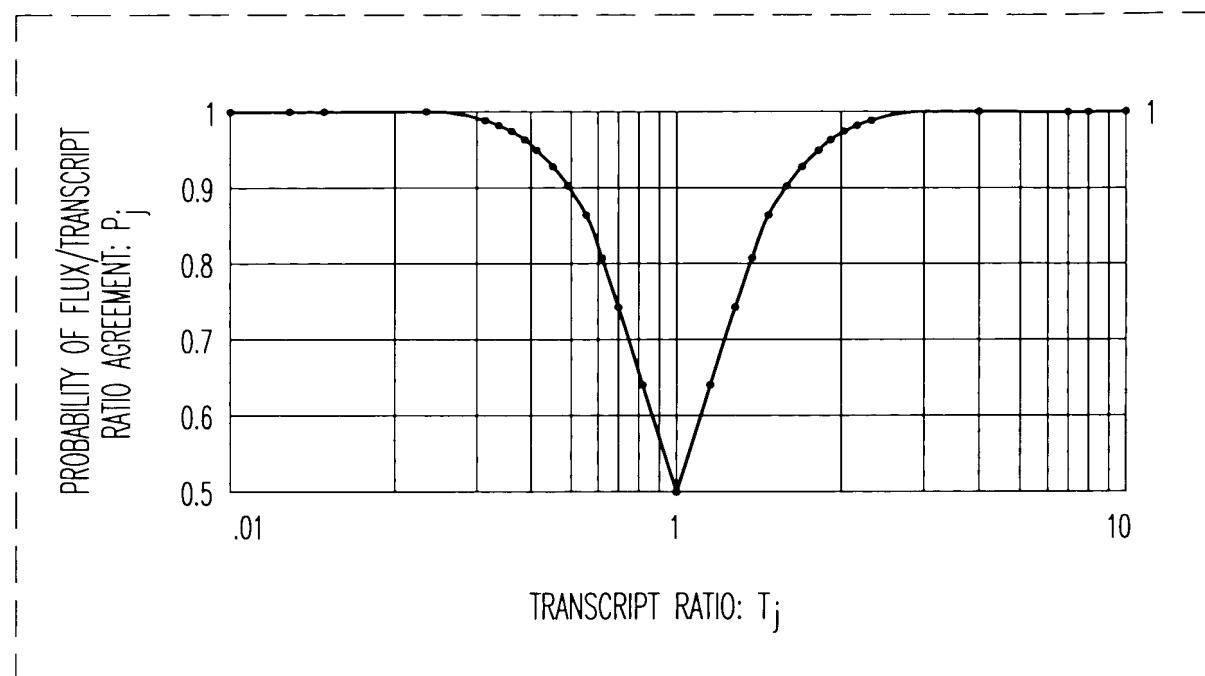


Fig. 7

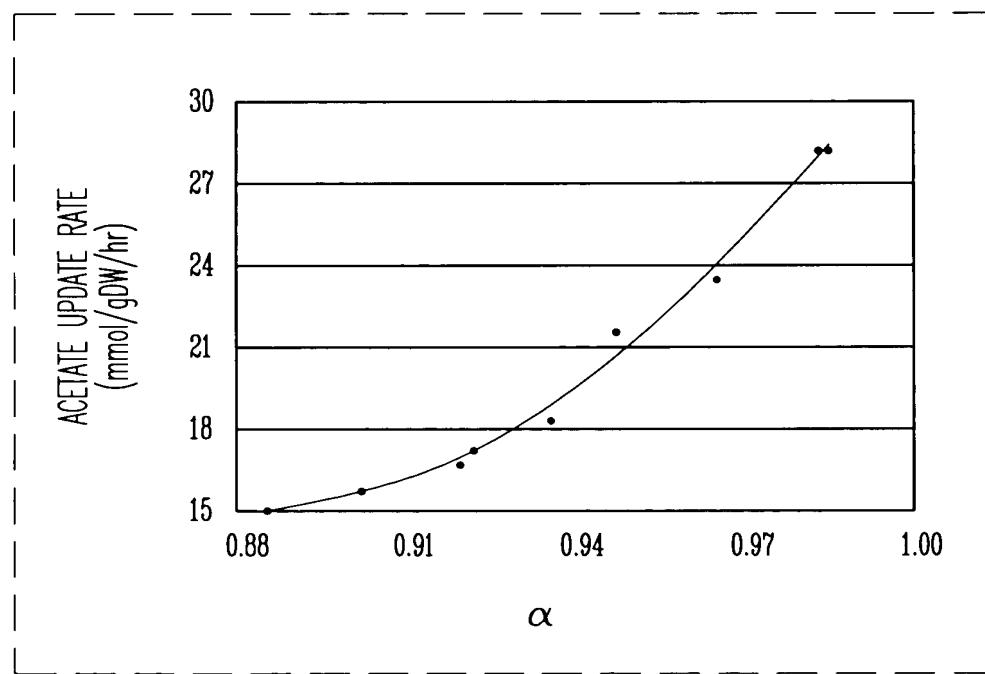


Fig. 8

MODEL PREDICTIONS
OF MAXIMUM THEORETICAL YIELDS OF AMINO ACIDS FOR
GROWTH ON GLUCOSE AND ACETATE

| | Maximum Theoretical Yield (mmol / per 10 mmol Glucose) | | | | Maximum Theoretical Yield (mmol / per 10 mmol Acetate) | | | |
|----------------------|---|--------------------------|--------------------|------------|---|--------------------------|--------------------|------------|
| | Palsson '93 | Modified Keasling '97 | Universal Model | % Increase | Palsson '93 | Modified Keasling '97 | Universal Model | % Increase |
| Alanine | 20.00 | 20.00 | 20.00 | - | 3.93 | 5.29 | 5.29 | - |
| Arginine | 7.74 | 9.26 | 10.07 | 8.75% | 1.51 | 2.43 | 2.65 | 9.05% |
| Asparagine | 15.60 | 18.18 | 19.23 | 5.77% | 3.24 | 4.66 | 4.91 | 5.45% |
| Aspartate | 18.20 | 20.00 | 20.00 | - | 3.82 | 5.29 | 5.29 | - |
| Cysteine | 9.75 | 11.49 | 11.90 | 3.57% | 1.81 | 3.29 | 3.42 | 3.80% |
| Glutamate | 10.00 | 13.33 | 13.33 | - | 2.68 | 3.65 | 3.65 | - |
| Glutamine | 10.00 | 13.33 | 13.33 | - | 2.50 | 3.46 | 3.46 | - |
| Glycine | 20.00 | 35.33 | 35.33 | - | 3.94 | 9.00 | 9.00 | - |
| Histidine | 7.30 | 9.77 | 9.80 | 0.23% | 1.37 | 2.43 | 2.54 | 4.53% |
| Isoleucine | 7.34 | 8.00 | 8.07 | 0.91% | 1.44 | 2.13 | 2.13 | - |
| Leucine | 6.67 | 8.00 | 8.00 | - | 1.59 | 2.18 | 2.18 | - |
| Lysine | 7.84 | 8.45 | 8.45 | - | 1.55 | 2.18 | 2.18 | - |
| Methionine | 5.74 | 7.04 | 7.19 | 2.16% | 1.11 | 1.81 | 1.85 | 2.46% |
| Phenylalanine | 5.29 | 5.76 | 5.76 | - | 1.00 | 1.47 | 1.47 | - |
| Proline | 10.00 | 10.91 | 10.91 | - | 2.10 | 2.90 | 2.90 | - |
| Serine | 20.00 | 23.04 | 23.04 | - | 3.94 | 5.87 | 5.87 | - |
| Threonine | 12.30 | 15.00 | 15.00 | - | 2.50 | 3.91 | 3.91 | - |
| Tryptophan | 4.14 | 4.67 | 4.73 | 1.28% | 0.76 | 1.17 | 1.19 | 1.32% |
| Tyrosine | 5.48 | 6.03 | 6.03 | - | 1.03 | 1.54 | 1.54 | - |
| Valine | 10.00 | 10.00 | 10.00 | - | 1.96 | 2.67 | 2.67 | - |

Palsson '93: *E. coli* model proposed by Palsson (1993)
 Modified Keasling '97: Modified Keasling (1997) *E. coli* model as described in text
 Universal Model: Modified Keasling (1997) *E. coli* model augmented with non-*E. coli* reactions
 compiled by the Kyoto Encyclopedia of Genes and Genomes
 % Increase: Between the modified Keasling (1997) model and the Universal model

Fig. 9

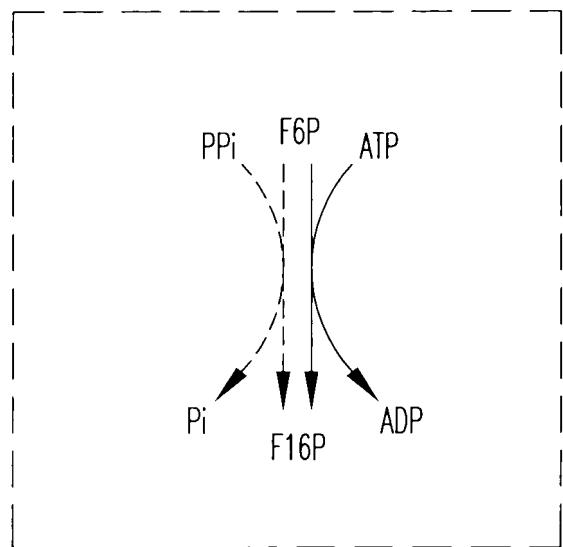


Fig. 10A

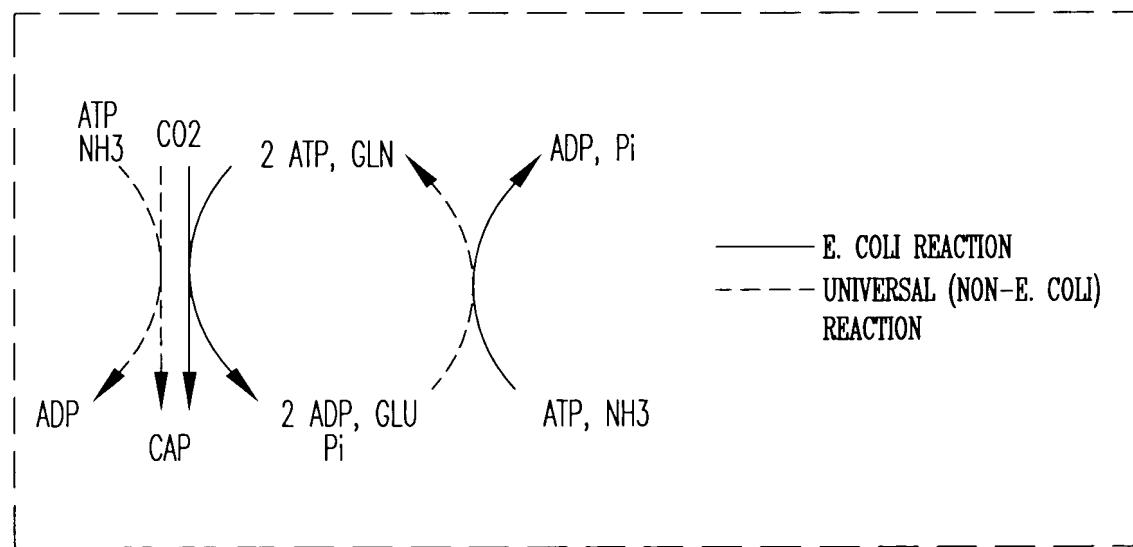


Fig. 10B

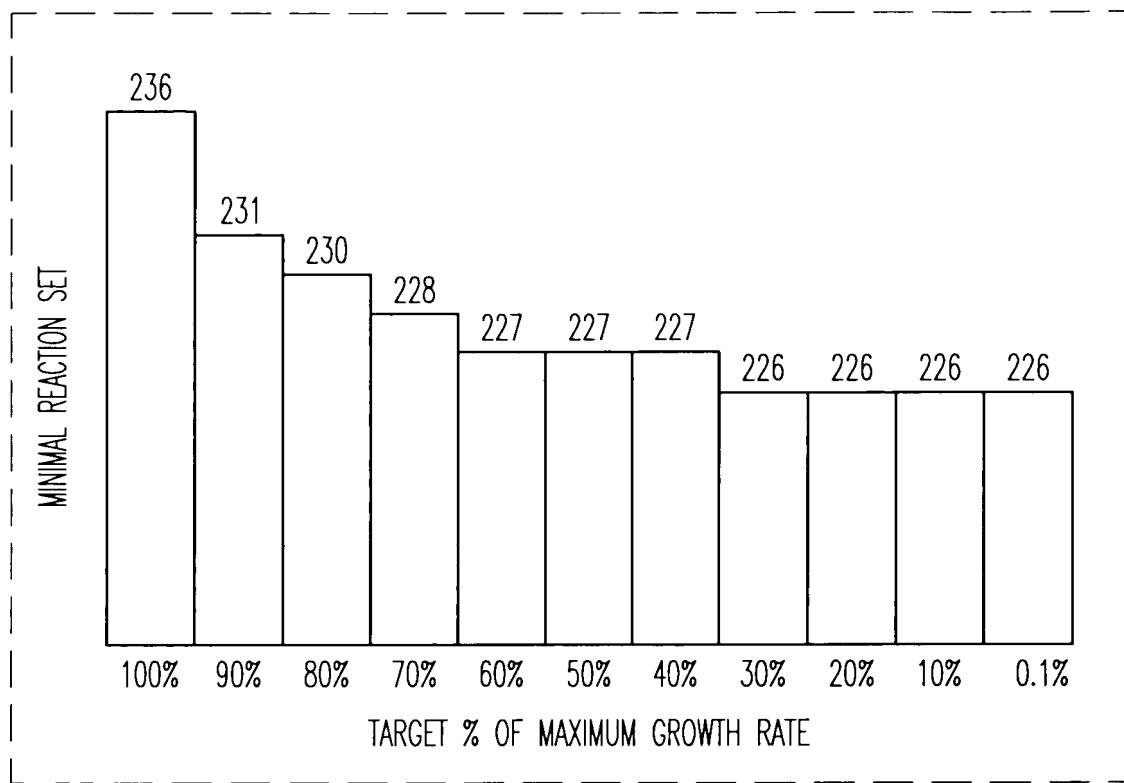


Fig. 11A

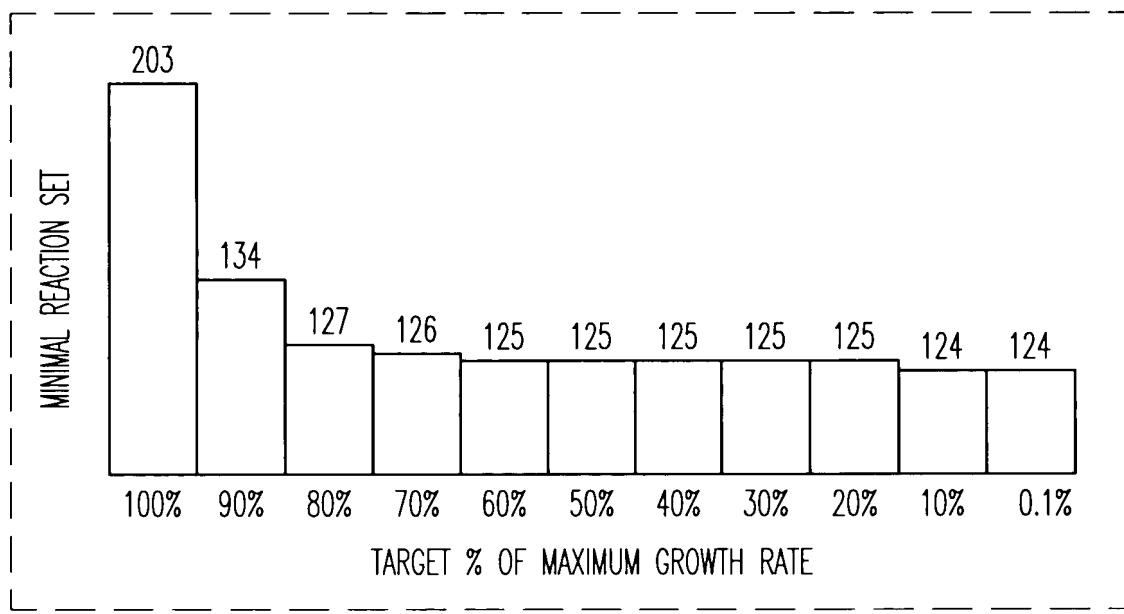


Fig. 11B

MODIFICATIONS TO THE PRAMANIK AND KEASLING MODEL*

| Enzymes | Reactions |
|---|---|
| Reactions assumed irreversible | |
| Phosphofructokinase | Fructose-1,6-bisphosphate → Fructose-6-phosphate + Pi |
| Citrate Synthase | Acetyl-CoA + Oxaloacetate → CoA + Citrate |
| 2-Ketoglutarate Dehydrogenase | 2-Ketoglutarate + NAD + CoA → Succinyl-CoA + CO ₂ + NADH |
| PRSCAIM Synthetase | RCAIM + ATP + Aspartate → ADP + Pi + PRSCAIM |
| Glycerol Kinase | Glycerol + ATP → Glycerol-3-phosphate + ADP |
| Reactions removed from model | |
| Unknown Pathway | 5'-methylthioadenosine → Adenosine + Methionine |
| Cystathione | Homocysteine + Adenosine ↔ s-Adenosyl-homocystine |
| Sulfotransferase | Adenosine-3,5-diphosphate + sulfite ↔ 3-Phosphoadenylylsulfate |
| Reactions modified | |
| Fructose-1,6-bisphosphate Aldolase | Fructose-1,6-bisphosphate → Fructose-6-phosphate + Pi |
| Isocitrate Dehydrogenase | Isocitrate + NADP ↔ CO ₂ + NADPH + 2-Ketoglutarate |
| Succinate Thiokinase | Succinyl-CoA + ADP + Pi ↔ ATP + CoA + Succinate |
| Prephenate Dehydrogenase | Prephenate + NAD → CO ₂ + NADH + para-Hydroxy phenyl pyruvate |
| Hol Dehydrogenase | Histidinol + 3 NAD → 3 NADH + Histidine |
| RCAIM Synthetase | AIR + CO ₂ + ATP → 5-p-Ribosyl-4-carboxy-5-aminoimidazole + ADP + Pi |
| GTP Cyclohydrolase | GTP → D6RP5P + Formate + PPi |
| 3,4-Dihydroxy-2-Butanone-4-Phosphate Synthase | Ribulose-5-phosphate → DB4P + Formate |
| H2Neopterin Triphosphate Pyrophosphatase | AHTD → PPi + Pi + DHP |
| CoA Synthase | OIVAL + METTHF + NADPH + ALA + CTP + 4 ATP + CYS → THF + NADP + AMP + 2 PPi + 2 ADP + CO ₂ + CoA + CDP |

MODIFICATIONS BASED ON INFORMATION BY KARP (1999)

Fig. 12

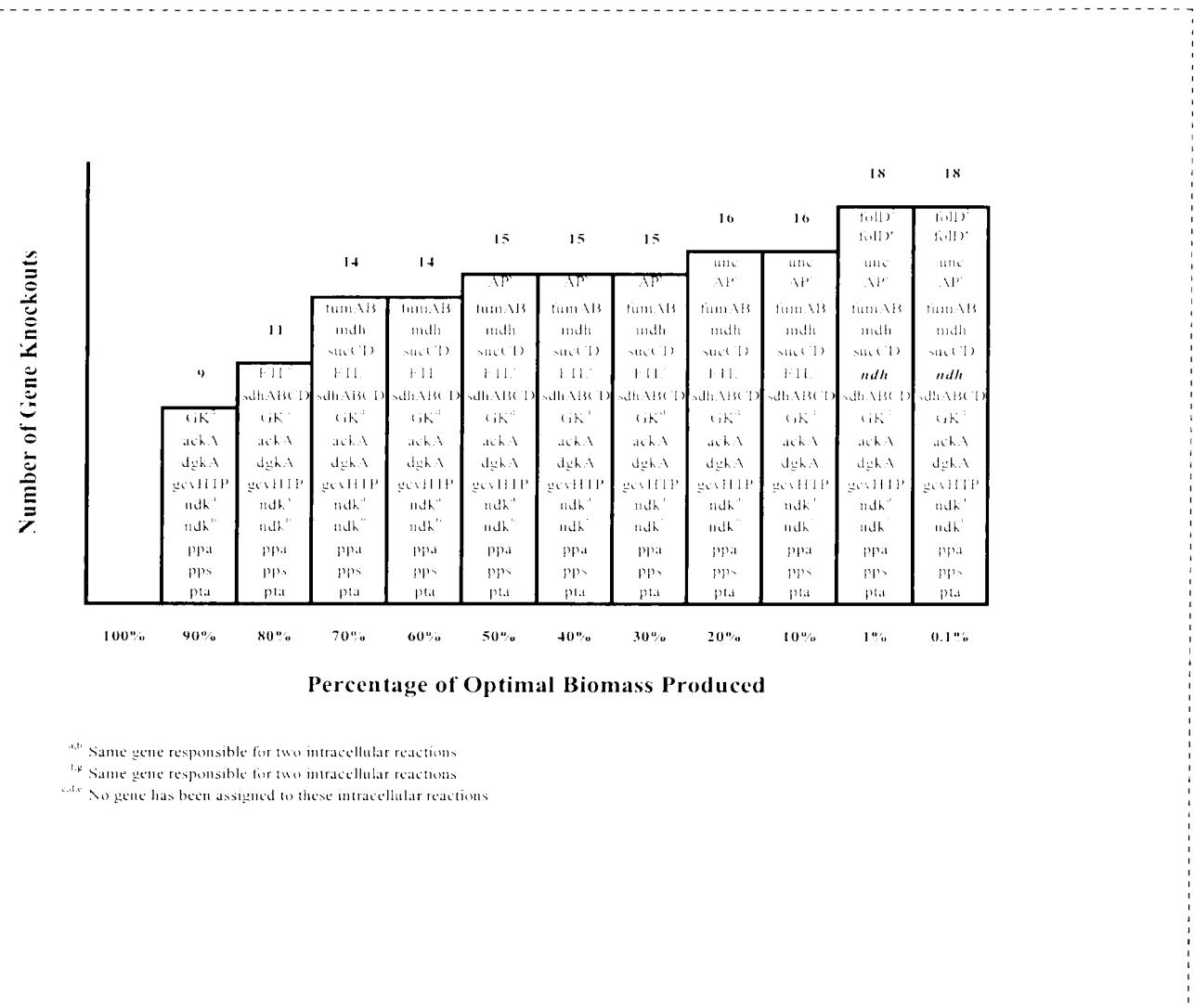


Fig. 13

GENES SELECTED FOR REMOVAL BY KNOCKOUT STUDY

| Enzymes | Genes | Reactions |
|-----------------------------|-------------------|---|
| 3,5-ADP Phosphatase | AP ^e | 35ADP → AMP + Pi |
| Acetate Kinase | ackA | AC + ATP → ACTP + ADP |
| CDP Kinase | ndk ^a | CDP + ATP → CTP + ADP |
| CMP Kinase | ndk ^b | CMP + ATP → CDP + ADP |
| F0F1-ATPase | unc | ADP + Pi + H _{ext} → ATP |
| Formate THF Ligase | FTL ^c | THF + FORMATE + ATP → ADP + Pi + FTHF |
| Fumarase | fumAB | FUM → MAL |
| Glyceraldehyde Kinase | GK ^d | GLAL + ATP → ADP + T3P1 |
| Glycine Cleavage System | gcvHTP | GLY + THF + NAD → METTHF + NADH + CO ₂ + NH ₃ |
| Malate Dehydrogenase | mdh | MAL + NAD → NADH + OA |
| Methenyl THF Cyclohydrolase | fold ^f | METHF → FTHF |
| Methylene THF Dehydrogenase | fold ^g | METTHF + NADP → METHF + NADPH |
| NADH Dehydrogenase I | ndh | NADH + Q → NAD + QH ₂ + 4 H _{ext} |
| PEP Synthase | pps | PYR + ATP → PEP + AMP + Pi |
| Phosphatidate Phosphatase | dgkA | DGR + Pi → PA |
| Phosphotransacetylase | pta | ACTP + COA → ACCOA + Pi |
| Pyrophosphatase | ppa | PPi → 2 Pi |
| Succinate Dehydrogenase | sdhABCD | SUCC + FAD → FADH ₂ + FUM |
| Succinate Thiokinase | sucCD | SUCCOA + GDP + Pi → GTP + COA + SUCC |

- a,b Same gene responsible for two intracellular reactions
- f,g Same gene responsible for two intracellular reactions
- c,d,e No gene has been assigned to these intracellular reactions

Fig. 14

MODEL SELECTIONS OF ENZYMATIC REACTIONS THAT WILL
 ENHANCE THE AMINO ACID PRODUCTION CAPABILITIES OF
ESCHERICHIA COLI

| Amino Acid | Substrate | EC# | Enzyme | Reaction Catalyzed |
|-------------------|----------------------|----------|---|---|
| Arginine | Glucose: | 2.7.1.90 | 6-Phosphofructokinase (pyrophosphate) | Fructose-6-P + PPi → Fructose-1,6-Bisphosphate + Pi |
| | | 2.7.2.2 | Carbamate kinase | ATP + NH3 + CO2 → ADP + Carbamoyl Phosphate |
| | Acetate: | 2.7.2.2 | Carbamate kinase | ATP + NH3 + CO2 → ADP + Carbamoyl Phosphate |
| | | 2.7.2.12 | Acetate kinase (pyrophosphate) | Acetate + PPi → Pi + Acetyl-Phosphate |
| Asparagine | Glucose/ Acetate: | 5.3.1.4 | Aspartate—ammonia ligase (ADP-forming) | ATP + NH3 + L-Aspartate → Pi + ADP + L-Asparagine |
| Cysteine | Glucose/ Acetate: | 2.7.7.5 | Sulfate adenylyltransferase (ADP) | Sulfate + ADP → Pi + Adenylyl-Sulfate |
| Histidine | Glucose: | 1.4.1.10 | Glycine dehydrogenase | NAD + glycine → glyoxylate + NADH + NH3 |
| | | 2.7.1.90 | 6-Phosphofructokinase (pyrophosphate) | Fructose-6-P + PPi → Fructose-1,6-Bisphosphate + Pi |
| | Acetate: | 1.4.1.10 | Glycine dehydrogenase | NAD + glycine → glyoxylate + NADH + NH3 |
| | | 4.1.1.38 | Phosphoenolpyruvate carboxykinase (pyrophosphate) | PPi + Oxaloacetate → CO2 + Pi + PEP |
| Isoleucine | Glucose: | many | | |
| Methionine | Glucose: | 2.7.7.5 | Sulfate adenylyltransferase (ADP) | Sulfate + ADP → Pi + Adenylyl-Sulfate |
| | Acetate: | 1.4.1.10 | Glycine dehydrogenase | NAD + glycine → glyoxylate + NADH + NH3 |
| | | 2.7.7.5 | Sulfate adenylyltransferase (ADP) | Sulfate + ADP → Pi + Adenylyl-Sulfate |
| | | 2.7.9.1 | Pyruvate,phosphate dikinase | Pyruvate + Pi + ATP → AMP + PPi + PEP |
| | | 4.1.1.38 | Phosphoenolpyruvate carboxykinase (pyrophosphate) | PPi + Oxaloacetate → CO2 + Pi + PEP |
| Tryptophan | Glucose: | 2.7.1.90 | 6-Phosphofructokinase (pyrophosphate) | Fructose-6-P + Ppi → Fructose-1,6-Bisphosphate + Pi |
| | | 2.7.9.1 | Pyruvate,phosphate dikinase | Pyruvate + Pi + ATP → AMP + PPi + PEP |
| | Acetate: | 2.7.9.1 | Pyruvate,phosphate dikinase | Pyruvate + Pi + ATP → AMP + PPi + PEP |
| | | 4.1.1.38 | Phosphoenolpyruvate carboxykinase (pyrophosphate) | PPi + Oxaloacetate → CO2 + Pi + PEP |

Fig. 15

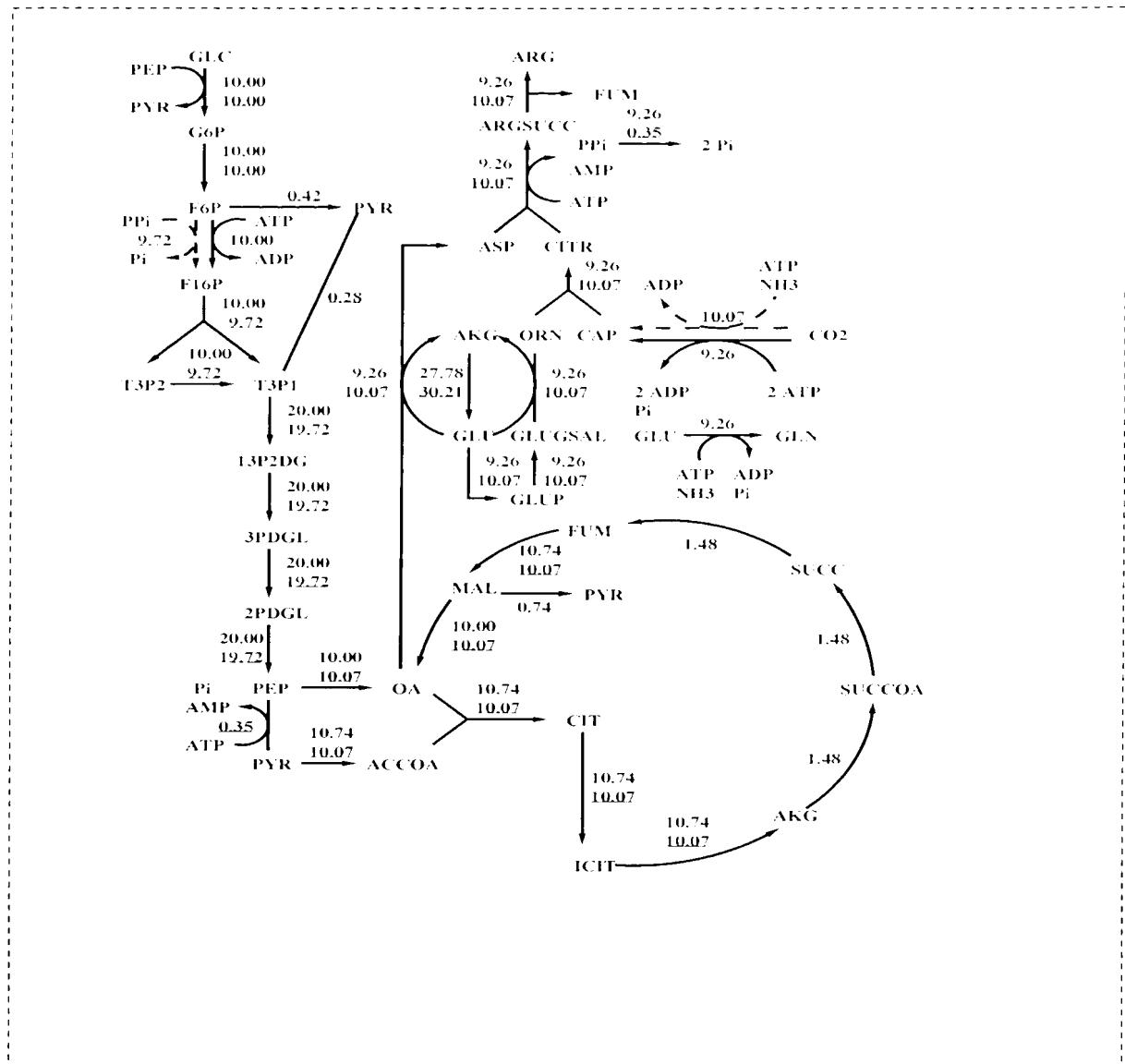


Fig. 16

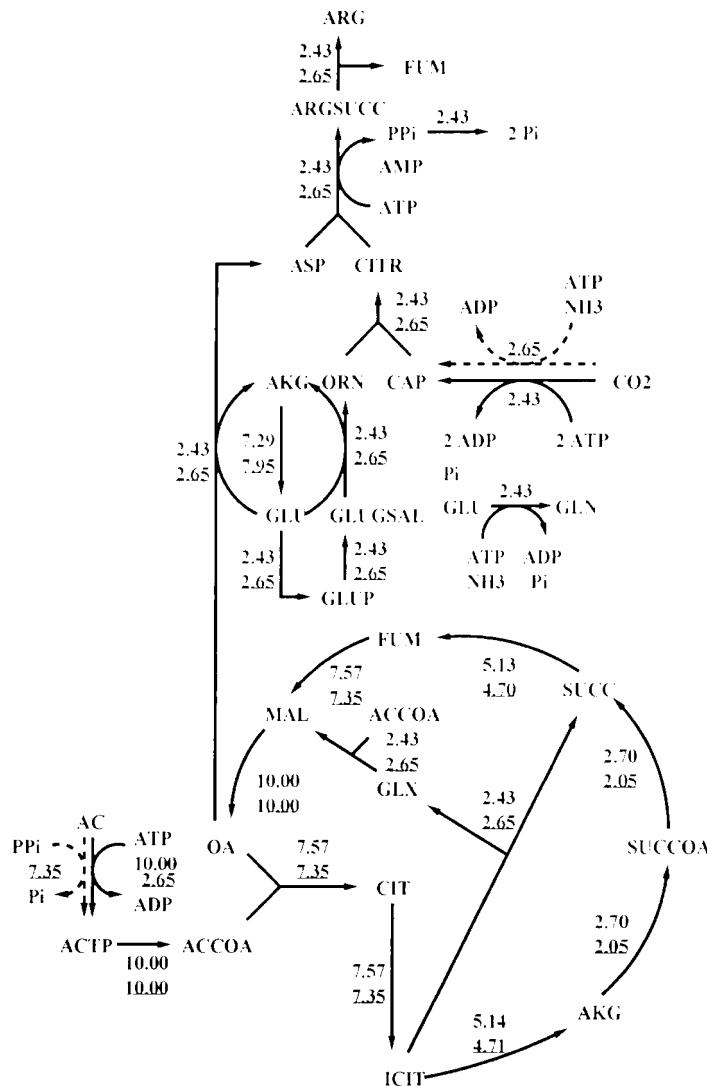


Fig. 17

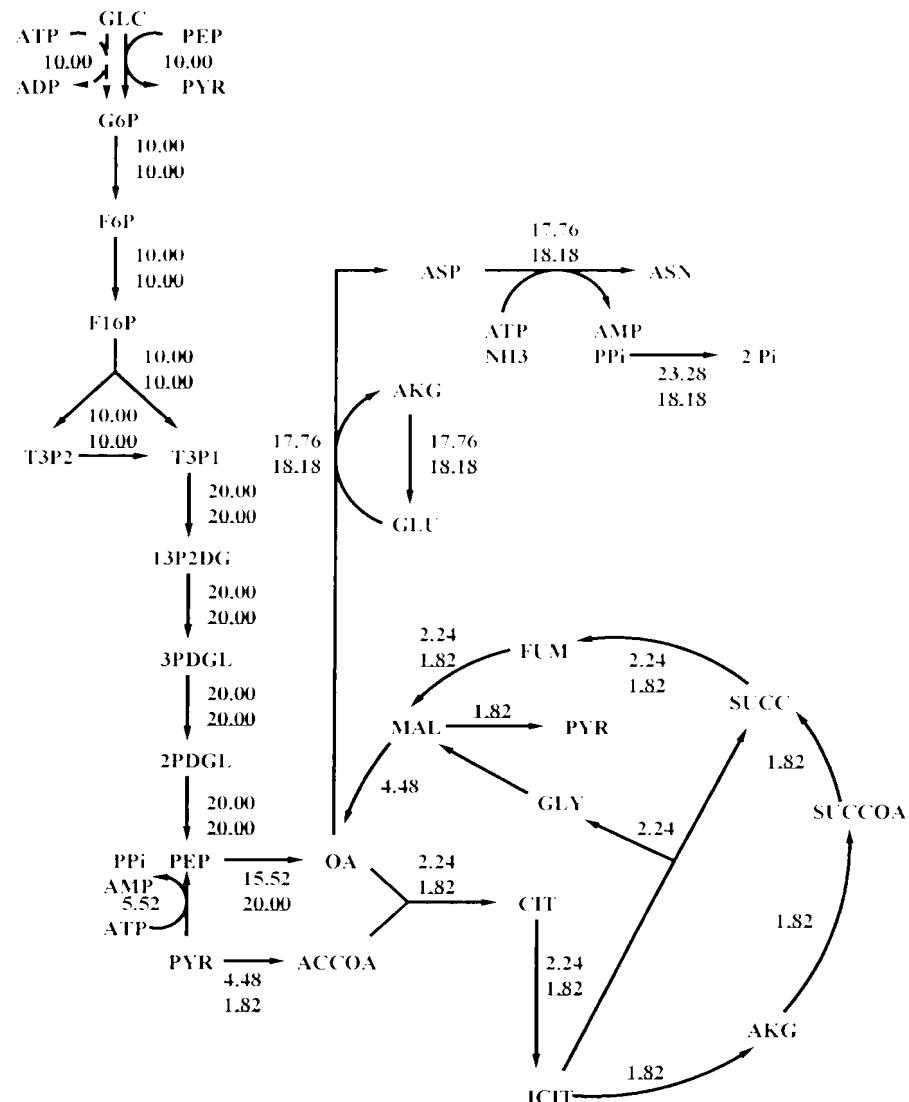


Fig. 18

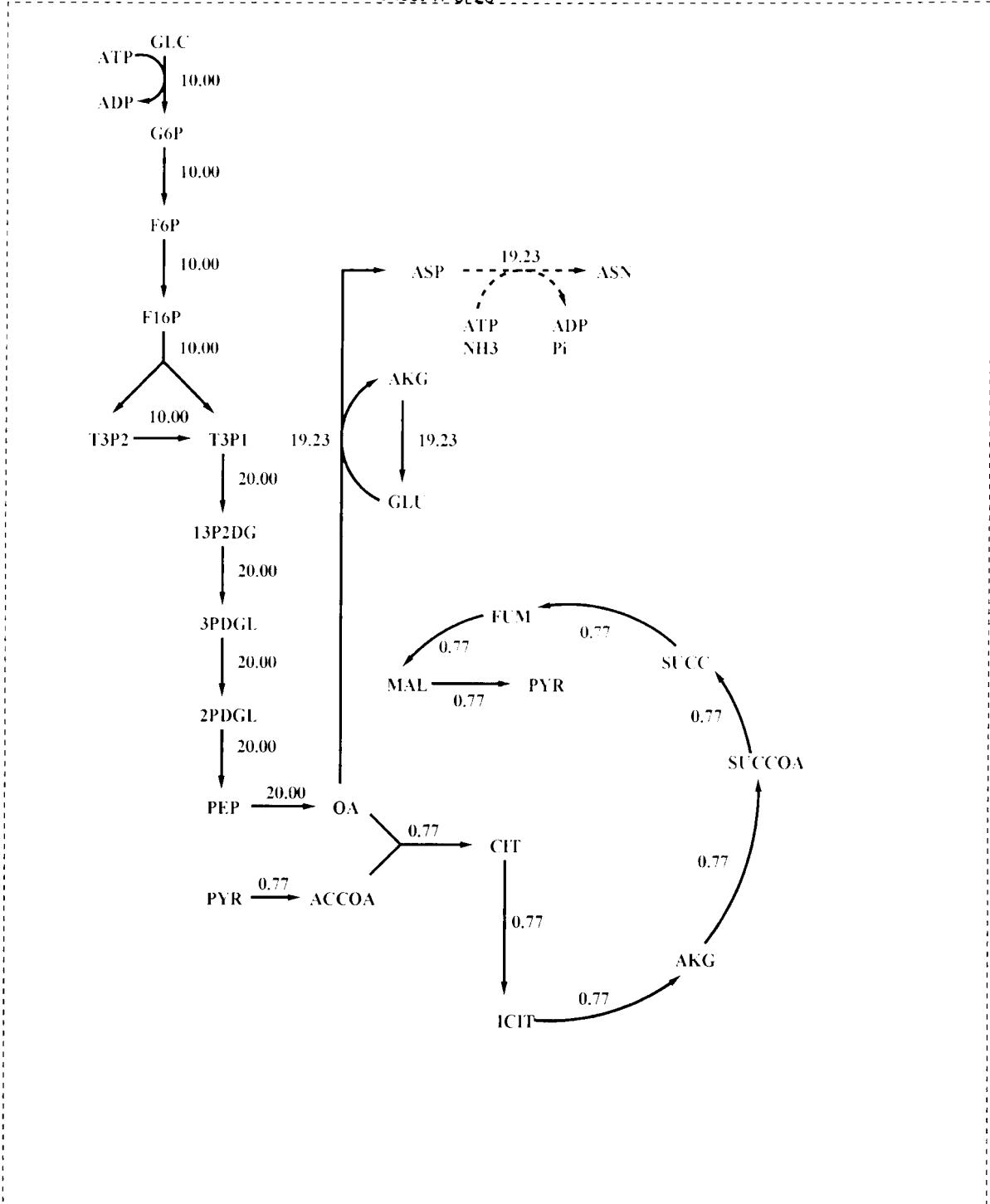


Fig. 19

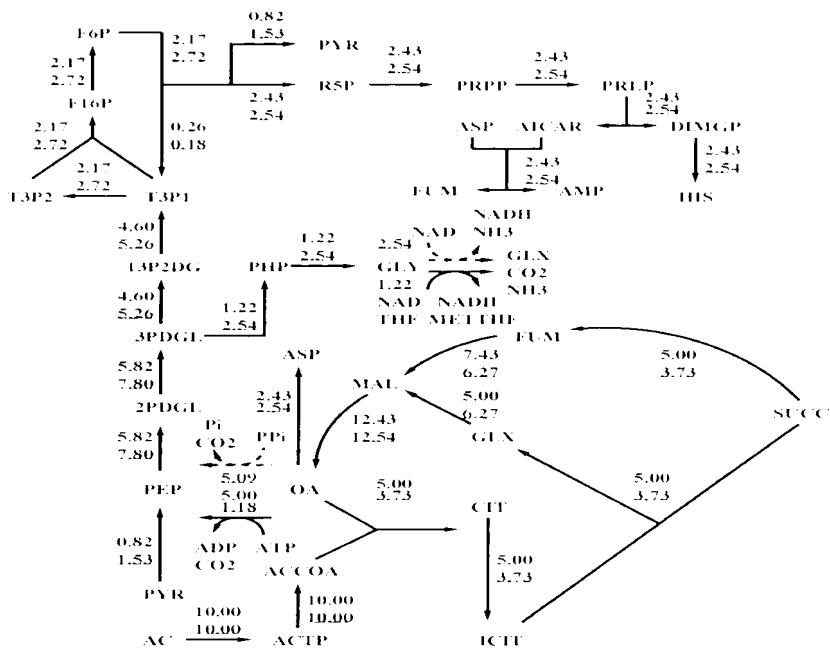


Fig. 20

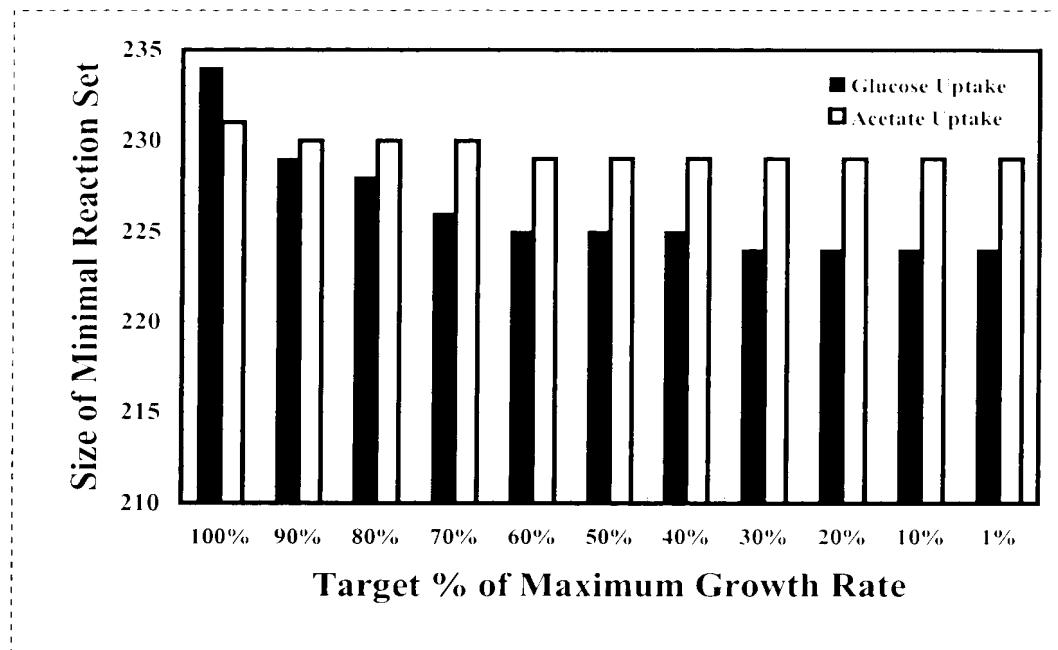


Fig. 21

**EVOLUTION OF MINIMAL REACTION SETS FOR CASE (I)
UNDER DECREASING GROWTH REQUIREMENTS.**

| Target % Maximum Growth Rate | Minimal Reaction Set (# Reactions) | Key Features |
|-------------------------------------|---|--|
| 100% | 234 | The glycolysis, tricarboxylic acid cycle, and pentose phosphate pathways are all operating in their forward directions, optimally generating the energy cofactors ATP, NADH, and NADPH required for cell growth. All available glucose is oxidized into the cell's only secreted byproduct, carbon dioxide. |
| 90% | 229 | The fluxes through two TCA cycle reactions 2-ketoglutarate dehydrogenase and succinate dehydrogenase are zero while succinyl-CoA synthetase operates in its reverse direction suggesting a less demanding energetic state under the submaximal growth demands. Acetate is now secreted as a byproduct along with carbon dioxide. |
| 80% | 228 | Fluxes through two additional TCA cycle reactions, fumarase and malate dehydrogenase, are eliminated while a reaction secreting fumarate is added. |
| 70% | 226 | The pentose phosphate pathway operates solely for nucleotide biosynthesis with the reaction fluxes through ribulose phosphate 3-epimerase, transketolase I, transketolase II, and transaldolase B all operating in reverse. Fluxes through glucose-6-phosphate dehydrogenase, lactonase, and 6-phosphogluconate dehydrogenase are absent in this case, replaced by pyridine nucleotide transhydrogenase which meets the cellular NADPH needs. In addition, formate is now secreted along with acetate, fumarate, and carbon dioxide. |
| 60%, 50%, 40% | 225 | Acetate is no longer secreted as a metabolic byproduct, but is converted to acetyl-CoA by acetyl-CoA synthetase. |
| 30%, 20%, 10%, 1% | 224 | Three glycolytic reactions, phosphoglycerate mutase, enolase, and pyruvate kinase are eliminated, but both serine deaminase and phosphoenolpyruvate synthase are added to supply the cell with phosphoenolpyruvate. |

Fig. 22

METABOLITES UPTAKEN OR SECRETED AT EACH TARGET GROWTH RATE ON AN OPTIMALLY ENGINEERED MEDIUM.

U DENOTES METABOLITE UPTAKE
 S DENOTES METABOLITE SECRETION

| Metabolite | Percentage of 100% Biomass Generation Required | | | | | | | | | | | | |
|-----------------------|--|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | 100% | 99.5% | 99% | 98% | 97% | 96% | 95% | 90% | 85% | 80% | 70% | 60% | 10% |
| Acetate | | | | | | | | | | | | S | S |
| Acetaldehyde | | | | | | | | | | | | U | |
| Adenine | | | U | U | U | U | U | U | U | U | U | U | |
| Adenosine | | | | | | | | | | | U | U | U |
| Alanine | | | | | | | | | | U | U | | |
| Arginine | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Asparagine | | | | | | | | | U | U | U | U | U |
| Aspartate | | | | | | | | | U | U | U | U | U |
| Carbon dioxide | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Cysteine | U | U | U | U | U | U | U | U | U | U | U | U | U |
| D-Alanine | | | | | | | | U | U | U | U | U | U |
| Thymidine | | U | U | U | U | U | U | U | U | U | U | U | U |
| Ethanol | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Glycerol | | | | | | | | | | | U | | |
| Glycerol-3-phosphate | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Glutamine | | | | | | | | | U | U | U | U | U |
| Glutamate | | | | | | | | | | | S | U | U |
| Glycine | | | | | | | U | U | U | U | U | U | U |
| Guanine | | | | U | U | U | U | U | U | U | | | |
| Guanosine | | | | | | | | | U | U | U | U | U |
| Histidine | | U | U | U | U | U | U | U | U | U | U | U | U |
| Isoleucine | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Leucine | | | | | | | U | U | U | U | U | U | U |
| Lysine | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Meso-diaminopimelate | | U | U | U | U | U | U | U | U | U | U | U | U |
| Methionine | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Mannitol | | | | | | | | | | | | U | U |
| Ammonia | U | U | U | U | U | U | U | U | U | | | | |
| Oxygen | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Phenylalanine | | | U | U | U | U | U | U | U | U | U | U | U |
| Phosphate | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Proline | | | | | | U | U | U | U | U | U | U | U |
| Putrescine | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Pyruvate | | | | | | | | | | U | U | U | U |
| Ribose | | | | | | | | | | | U | U | |
| Serine | | | | | | | | | U | U | U | U | U |
| Spermidine | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Threonine | | U | U | U | U | U | U | U | U | U | U | U | U |
| Tryptophan | U | U | U | U | U | U | U | U | U | U | U | U | U |
| Tyrosine | | | U | U | U | U | U | U | U | U | U | U | U |
| Uracil | | | | | | | U | U | U | U | U | U | U |
| Uridine | | | | | | | | | | U | U | U | U |
| Valine | | | | | | | | U | U | U | U | U | U |
| # Metabolites Uptaken | 12 | 17 | 19 | 21 | 22 | 24 | 26 | 28 | 29 | 31 | 29 | 34 | 34 |

Fig. 23

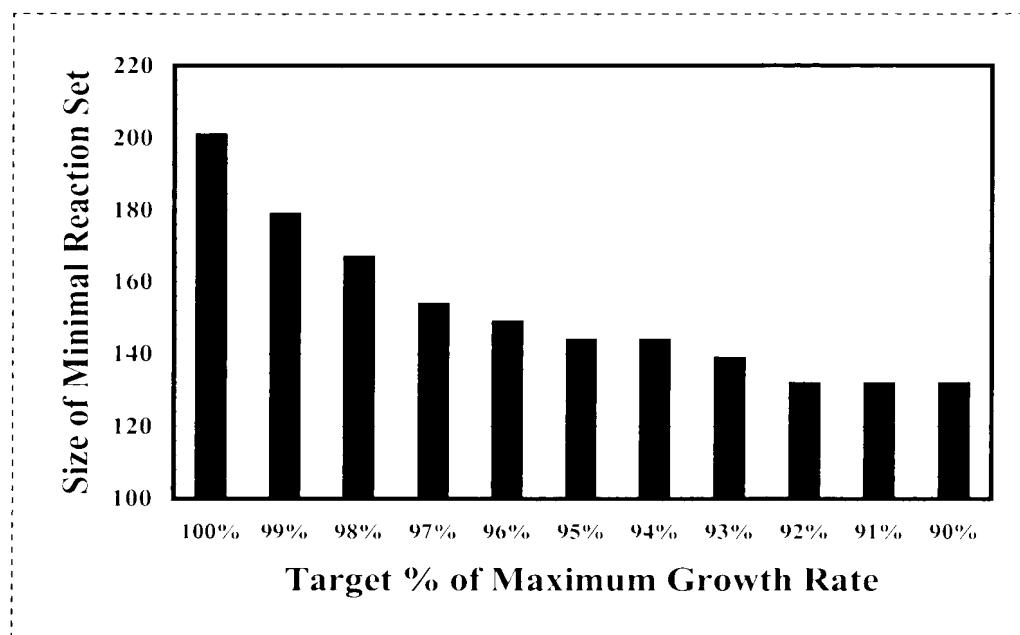


Fig. 24

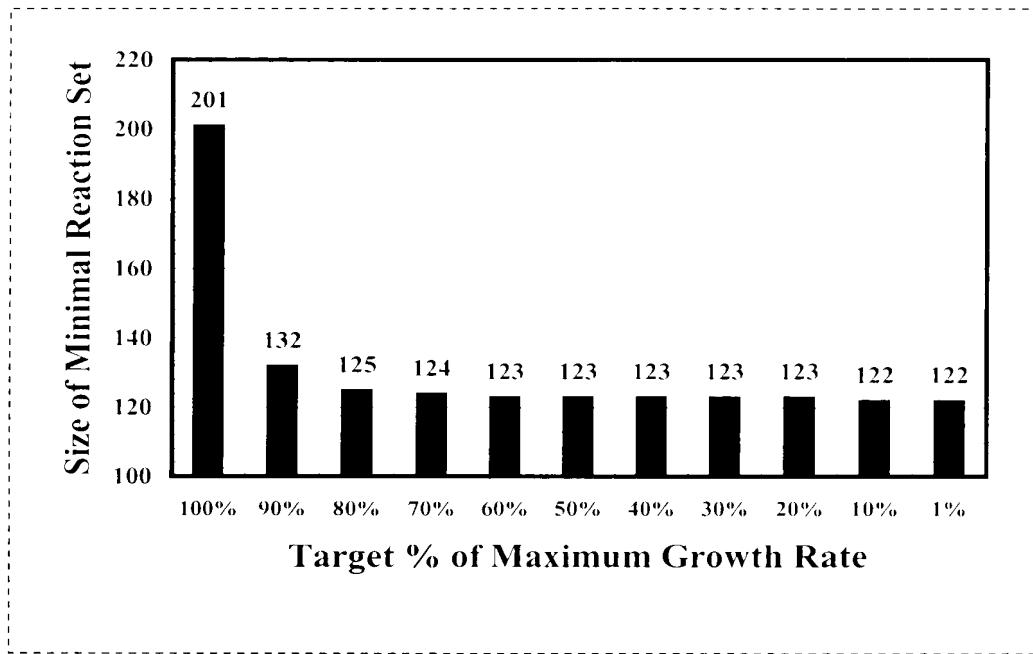


Fig. 25

EVOLUTION OF MINIMAL REACTION SETS FOR CASE (II) UNDER DECREASING GROWTH REQUIREMENTS.

| Target % Maximum Growth Rate | Minimal Reaction Set (# Reactions) | Key Features |
|------------------------------|------------------------------------|--|
| 100% | 201 | The organic material transported into the cell includes ethanol and glycerol-3-phosphate which fuel glycolysis, the TCA cycle, and PPP. The flux directions of the glycolysis pathway are split with all reaction fluxes preceding glyceraldehyde-3-phosphate (G3P) dehydrogenase operating in reverse, and all fluxes following and including G3P dehydrogenase operate in their forward directions. Putrescine, spermidine, and five amino acids are transported into the network eliminating the need for biosynthetic pathways for these components. |
| 90% | 132 | While the PPP and TCA cycle reactions are still functional, the network no longer utilizes the five glycolytic reactions from glyceraldehyde-3-phosphate dehydrogenase to pyruvate kinase. Consequently, the TCA cycle is completely fueled by imported ethanol and acetate rather than flux from the glycolysis pathway. |
| 80% | 125 | This network tolerates the complete elimination of the TCA cycle and glyoxylate shunt. As a result, the function of the pentose phosphate pathway reactions is no longer restricted to nucleotide biosynthesis, but now includes the formation of cellular NADPH. Most of this NADPH is subsequently converted to NADH by pyridine nucleotide transhydrogenase to replace the cellular reducing power lost from the inactivity of the TCA cycle. |
| 70% | 124 | A slightly less efficient set of internal metabolic reactions enables the growth demands to be met with the importation of one less metabolite (i.e. one less transport reaction) than its 80% counterpart. |
| 60% 50%, 40% 30%, 20% | 123 | Neither the TCA cycle nor PPP are utilized for reducing power. Most of the cellular reducing capabilities are now generated from the uptake of ethanol and its subsequent conversion into acetyl-CoA. |
| 10%, 1% | 122 | This minimal network is comprised mostly of cell envelope and membrane lipid biosynthetic reactions, along with a number of transport and salvage pathway reactions. Here, the three core metabolic routes, glycolysis, the TCA cycle, and the pentose phosphate pathway are almost completely dismantled with only one glycolytic and 4 PPP reactions remaining. |

Fig. 26

FUNCTIONAL CLASSIFICATION OF MINIMAL
NETWORKREACTIONS FOR GROWTH ON AN OPTIMALLY
ENGINEERED MEDIUM.

| Functional Classification# rxns | |
|---------------------------------|-----|
| ALA Isomerization | 1 |
| Alternative Carbon Source | 7 |
| Anaplerotic Reactions | 1 |
| Cell Envelope | |
| Biosynthesis | 29 |
| EMP Pathway | 5 |
| Membrane Lipid | |
| Biosynthesis | 16 |
| Pentose Phosphate | |
| Pathway | 4 |
| Pyrimidine Biosynthesis | 1 |
| Respiration | 5 |
| Salvage Pathways | 17 |
| Transport | 36 |
| | 122 |

Fig. 27

COMPARISON OF MINIMAL METABOLIC GENE/REACTION SETS BASED ON FUNCTIONAL CLASSIFICATION*

| Metabolic Function | Essential Gene Set <i>Ref. (2)</i> | Minimal Gene Set <i>Ref. (5)</i> | Minimal Reaction Set |
|--|--|--|----------------------------|
| | # Genes | # Genes | # Reactions |
| Amino acid biosynthesis | 0 | 0 | 1 |
| Biosynthesis of cofactors, prosthetic groups, and carriers | 4 | 3 | 0 |
| Cell envelope | 2 | 11 | 29 |
| Central intermediary metabolism | 7 | 7 | 1 |
| Energy metabolism | 31 | 32 | 21 |
| Fatty acid and phospholipid metabolism | 5 | 7 | 16 |
| Purines, pyrimidines, nucleosides, and nucleotides | 17 | 14 | 18 |
| Transport and binding proteins | 17 | 25 | 36 |
| | 83 | 99 | 122 |

Fig. 28